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Estrogen receptor α , but not β , is required for optimal dendritic cell differentiation and CD40-induced cytokine production¹

Short title: Estrogen receptor α is required for optimal dendritic cell development

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Nonstandard abbreviations used in this paper : E2, 17 β -estradiol; ER, estrogen receptor; BM, bone marrow; CM, conventional medium containing phenol red and regular FCS; SFM, steroid-free medium.

Keywords: Dendritic cells; Cell Differentiation; Estrogen Receptors; Knockout mice

Abstract

Dendritic cells (DC) are critical actors in the initiation of primary immune responses and regulation of self-tolerance. The steroid sex hormone 17 β - α estradiol (E2) has been shown to promote the differentiation of DCs from bone marrow (BM) precursors *in vitro*. However, the estrogen receptor (ER) involved in this effect has not yet been characterized. Using recently generated ER α - or ER β -deficient mice, we investigated the role of ER isotypes in DC differentiation and acquisition of effector functions. We report that estrogen-dependent activation of ER α , but not ER β , is required for normal DC development from BM precursors cultured with GM-CSF. We show that reduced numbers of DCs were generated in the absence of ER α -activation and provide evidence for a cell autonomous function of ER α -signaling in DC differentiation. ER α -deficient DCs were phenotypically and functionally distinct from wild-type DCs generated in the presence of estrogens. In response to microbial components, ER α -deficient DCs failed to upregulate MHC class II and CD86 molecules, which could account for their reduced capacity to prime naive CD4⁺ T lymphocytes. Although, they retained the ability to express CD40 and to produce pro-inflammatory cytokines (e.g. IL-12, IL-6) upon TLR engagement, ER α -deficient DCs were defective in their ability to secrete such cytokines in response to CD40-CD40L interactions. Combined, these results provide the first genetic evidence that ER α is the main receptor regulating estrogen-dependent DC differentiation *in vitro* and acquisition of their effector functions.

Introduction

Dendritic cells (DC) are the major class of antigen presenting cells (APC). They play a central role in the initiation and coordination of the innate and adaptive immune responses by integrating signals from pathogens, cytokines and T cells. DC activation can be induced by a variety of signal, such as microbial or viral products which are directly recognized by members of the Toll-like receptor (TLR) family (1). Upon activation, DCs mature into potent APCs expressing high levels of MHC molecules and costimulatory molecules (CD80/CD86, CD40) and secrete immunomodulatory cytokines, such as IL-12, IL-6 and IL-10 that control the expansion and differentiation of naive T cells into effectors (2-4). Although, IL-12 synthesis by DCs can be initiated by microbial signals, it requires reciprocal signaling from T cells for optimal production (5, 6). This cellular dialogue is mainly dependent on the interactions between CD40 expressed by DCs and its ligand CD154 (CD40L) which is expressed by CD4⁺ T cells following TCR stimulation (7).

DCs represent an extremely plastic and versatile cell type, which plays crucial role not only in the initiation and control of immunity and tolerance, but can also contribute to the induction of pathological situations such as autoimmune diseases (8). Although sex-based differences in the susceptibility to autoimmune diseases are well known, the underlying mechanisms are not understood (9). It has been shown that sex hormones, particularly estrogens may contribute to the pathogenesis of some autoimmune diseases (9). The identification of estrogen receptors (ER) on immune cells suggested that sex steroid hormones, such as estrogens, may act directly on the immune system, modulating APC functions, lymphocyte activation and/or cytokine-gene expression. Estrogen receptors α (ER α) and β (ER β) belong to the nuclear receptor family of transcription factors. They are encoded by two different genes *Esr1* and *Esr2* and account for most of the known effects of estrogens (10). Human and mouse DCs express transcripts for both ER isotypes (11, 12) and

could therefore represent a critical target for estrogens *in vivo*. Indeed, it has been shown that differentiation of DCs from murine bone marrow (BM) cells in the presence of GM-CSF was dramatically dependent on the presence of estrogens normally found in conventional culture medium (12). However, direct evidence for a role of ER α and/or β -signaling in this effect was still lacking.

In this study, we have attempted to elucidate the respective role of ER α and ER β on GM-CSF-induced DC development and acquisition of effector functions, using recently generated ER-deficient mice (13). We confirmed the requirement for estrogens to generate optimal number of fully functional DCs *in vitro*, and we demonstrated that E2 effect on DC differentiation was dependent on ER α but not ER β activation. The quantitative defect in DC development observed in the absence of ER α -signaling was also associated with phenotypic and functional differences, as assessed by expression of maturation markers, ability to stimulate T cell proliferation or to secrete pro-inflammatory cytokines in response to TLR- or CD40-dependent stimulations. Together these results show that E2-dependent activation of ER α , but not ER β , regulates critical steps involved in the development and acquisition of effector functions of DCs.

Materials and methods

Mice

Female C57BL/6 (B6) (H-2^b, CD45.2) mice were purchased from Centre d'Elevage R. Janvier (Le Genest St Isle, France). ER α -deficient B6 mice (CD45.2) which have a deletion in the exon 2 of the ER- α gene (ER α ^{-/-}), ER- β -deficient B6 mice (ER β ^{-/-}) and littermate controls on B6 background have been previously described (13). Females were used in most experiments with ER-mutant mice but identical results were obtained with males. CD45.1 B6.SJL congenic mice were initially obtained from The Jackson Laboratory (Bar Harbor, ME). B10.D2 ER α ^{-/-} (H-2^d) mice were generated in our own animal facilities by crossing ER α ^{+/-} B6 mice with B10.D2 mice obtained from Harlan (UK). After three back-crosses on B10.D2 background, ER α ^{+/-} H-2^{d/d} homozygotes were selected to generate B10.D2 ER α ^{-/-} or ER α ^{+/+} female mice. DO11.10 transgenic mice carrying a V α 2/V β 8 TCR specific for OVA323-339/I-A^d complexes (14) on BALB/c (H-2^d) background were initially provided by Dr L. Adorini (Bioxell, Milan, Italy). Mice were bred and maintained in our specific pathogen-free animal facility. Protocols were approved by our institutional review board for animal experimentation.

DC generation from murine bone-marrow.

Bone-marrow derived dendritic cells (BMDC) were generated as previously described (15). Briefly, BM cells were flushed out from femurs and tibias. After lysis of red blood cells in ammonium chloride potassium (ACK), BM cells were cultured in conventional medium or steroid-free medium containing 20 ng/ml murine GM-CSF (PeproTech, London, UK) at 2×10^5 cells/ml in bacteriological petri dish (Greiner Bio-One, Poitiers, France). On day 3, an equal volume of fresh medium with 20 ng/ml GM-CSF was added to the culture and on day 6, half of the medium was removed and replaced by fresh medium containing 10 ng/ml GM-

CSF. Conventional medium (referred as CM) was RPMI 1640 (Eurobio, Courtabœuf, France) supplemented with 10 % heat-inactivated FCS (ATGC Biotechnologie, Noisy Le Grand, France), 1 mM sodium pyruvate, 1 % non-essential amino acids, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol and 50 μ g/ml gentamicin (Sigma Aldrich, Saint Quentin Fallavier, France). Culture medium used for experiments in estrogen controlled conditions (referred as SFM) contained phenol red-free RPMI 1640 (Eurobio, Courtabœuf, France) with 10 % dextran charcoal-treated FCS (Hyclone, Utah, USA) supplemented with 1 mM sodium pyruvate, 1 % non-essential amino acids, 2 mM L-glutamine, 50 μ M 2-mercaptoethanol and 50 μ g/ml gentamicin (Sigma Aldrich). Cell treatments with 17 β -estradiol (E2) (Sigma Aldrich), with the ER antagonist ICI_{182,780} (Tocris, MO, US) or with DMSO vehicle were performed at days 0, 3 and 6 of the cultures. Total cells in the culture were recovered at day 8 or day 9 and counted. DC yield was calculated by multiplying total cell number by the percentage of CD11c⁺Gr-1⁻ DC in the culture which was determined by flow cytometry as described below.

For mix bone marrow cultures, BM cells from CD45.1 mice (10⁵ cells/ml) were mixed with equal amount of CD45.2 ER α ^{+/+} or CD45.2 ER α ^{-/-} BM cells (10⁵ cells/ml) and cultured with GM-CSF as described previously. Expressions of CD45.1 and CD45.2 alloantigens and of CD11c and Gr-1 markers were assessed by flow cytometry to calculate DC yields from each CD45 allotypes.

DC purification and stimulations

DCs were purified from GM-CSF cultures by positive CD11c selection by preincubation with CD11c-specific microbeads and subsequent immunomagnetic sorting using minimacs columns (Miltenyi Biotec, Paris, France). Purity after enrichment was routinely between 80 to 95 % CD11c⁺ cells as assessed by flow cytometry. For stimulations

with TLR agonists, purified DCs were stimulated with LPS (*Escherichia coli* 0111:B4 LPS Ultra-Pure (Invivogen, Toulouse, France), poly I:C (Sigma Aldrich), CpG-ODN phosphorothioate oligodeoxynucleotide 1668 (Sigma Aldrich) or GpC-ODN control (Sigma Aldrich). For CD40-dependent stimulation, purified DCs were co-cultured with control mock-transfected or CD40L (CD154)-expressing NIH 3T3 fibroblasts which were a gift of Dr. P. Hwu (National Cancer Institute, Bethesda, MD) and were kindly provided by Dr. C. Reis e Sousa (Cancer Research UK, London).

Analysis of surface markers and cytokine production

Before staining, cells ($5-10 \times 10^5$) were incubated 15 min at room temperature with blocking buffer (PBS with 1 % FCS, 3 % normal mouse serum, 3 % normal rat serum, 5 mM EDTA, 1 % NaN₃) containing 5 µg/ml anti-CD16/CD32 (2.4G2, ATCC). For surface cell staining, cells were incubated for 30 min on ice with FITC-, PE-, biotin- or APC-conjugated monoclonal antibodies (mAbs) diluted at the optimal concentration in FACS buffer (PBS 1 % FCS, 5 mM EDTA, 1 % NaN₃). When biotinylated mAbs were used, cells were washed twice in FACS buffer before incubation with APC conjugated streptavidin (eBioscience, San Diego, CA). The following mAbs for cell surface staining were purchased from BD Biosciences (San Jose, CA) : anti-CD11c (HL3), anti-CD11b (M1/70), anti-Ly6C (AL-21), anti-CD86 (GL1), or from eBioscience: anti-CD11c (N418), anti-MHC class II (M5/114.15.2), anti-CD40 (HM40-3), anti-CD45.1 (A20), anti-CD45.2 (104), anti-TLR4/MD2 (MTS510) or anti-CD4 (GK1.5). Flow cytometry analysis were performed on a FACSCalibur flow cytometer (BD Biosciences).

For phenotypic analysis of DC maturation and intracellular cytokine production, purified DCs were incubated in CM supplemented with 10 ng/ml GM-CSF and stimulated for 18 h with 2 µg/ml LPS. DCs were recovered by incubation for 15 min on ice with PBS containing 1 % FCS and 2 mM EDTA. For detection of intracellular cytokine production,

DCs stimulated as indicated above were incubated with 10 µg/ml brefeldin A (Sigma Aldrich) for the last 4 h of culture. After surface staining with FITC-anti-MHCII and APC-anti-CD11c and fixation in PBS 1% paraformaldehyde, cells were permeabilized with 0.5 % saponin and intracellular cytokine staining was performed with PE-anti-IL-6 (MP5-20F3), FITC-anti-TNF-α (MP6-XT2), PE-anti-IL-12p40/p70 (C15.6) or PE-rat IgG1 isotype control, all from BD Biosciences.

For cytokine production, DCs were cultured in 96-well plates (3×10^4 cells/well) and stimulated with 2 µg/ml of LPS, 10 µg/ml poly I:C, 1 µg/ml CpG-ODN or 1 µg/ml GpC-ODN control. For CD40-dependent stimulation, DCs (6×10^4 cells/well) were co-cultured with CD40L-transfected NIH-3T3 fibroblasts (2.5×10^4 cells/well) in 96-well plates in the absence or presence of anti-CD154 mAb (BD Biosciences). Mock-transfected NIH-3T3 were used as control. To assess IL-12p70 production, 5 ng/ml IFN-γ (PeproTech, London, UK) were added to the stimulations. Production of IL-6, TNF-α and IL-12p40 were measured in 24 h culture supernatants and IL-12p70 in 48 h culture supernatants. Cytokines were quantified by two site sandwich ELISA (all mAbs were purchased from BD Biosciences).

Assessment of antigen-specific CD4⁺ T cell activation

The ability of DCs to activate Ag-specific T cells was monitored by measuring CFSE dilution and thymidine incorporation of OVA-specific CD4⁺ T cells from DO11.10 TCR transgenic mice. CD4⁺ T cells were enriched by negative selection using CD4⁺ T cell isolation kit (DynaL Biotech, Compiègne, France) and labeled with 5 µM CFSE as described elsewhere (16). CFSE labeled DO11.10 CD4⁺ T cells were incubated at 1×10^5 cells per well in 96-well plates (Costar) with a constant number of CD11c-sorted ERα^{-/-} or ERα^{+/+} B10.D2 DCs (3×10^4 cells) per well and titrated concentrations of endotoxin-free OVA protein (Sigma) or OVA₃₂₃₋₃₃₉ peptide (NeoMPS, Strasbourg, France). Cells were cultured in CM at 37°C in a

humidified atmosphere containing 5 % CO₂. After 72 h culture, cell division was assessed by flow cytometry. DO11.10 TCR transgenic CD4⁺ T cells labeled with CFSE were stained with biotinylated anti-DO11.10 clonotype KJ1.26 and PE conjugated anti-CD4. To assess CD4⁺ T cell proliferation, cultured were set up as above and pulsed with 1 µCi ³H-TdR (40 Ci/nmol, the Radiochemical Centre, Amersham, UK) at 48 h. Incorporation of ³H-TdR was measured 12 h later by using a MicroBeta TriLux luminescence counter (Perkin Elmer, Waltham, MA, USA).

Results

Generation of DC from BM progenitors is impaired in absence of E2 and requires ER α -, but not ER β -dependent signaling.

Culture of BM cells in the presence of GM-CSF leads to the differentiation of CD11c⁺ myeloid DCs, expressing CD11b and high to intermediate levels of MHC class II molecules (15, 17). Using this culture system, it has been previously shown that the absence of estrogens or the presence of ER antagonists resulted in an impaired development of CD11c⁺ CD11b^{int} DCs that normally represent the majority of cells generated in estrogen-supplemented medium (12). Instead, culture of BM cells in steroid-hormone deficient medium generated mainly CD11c negative cells that express the myeloid differentiation marker Gr-1 and low to high levels of CD11b (12). In the present study, we used this culture system to determine the role of ER-isotypes in this effect of E2 on DC differentiation using recently generated ER α - or ER β -deficient mice (13). BM cells from ER α ^{-/-} or ER α ^{+/+} littermate control mice were culture in conventional medium (CM) in the presence or absence of the pure ER antagonist ICI_{182,780}. As shown in Fig. 1, the frequency as well as the absolute number of CD11c⁺ Gr-1⁻ DCs that developed from ER α ^{-/-} BM cultures was reduced up to 3 to 4-fold as compared to wild-type BM. This quantitative defect was associated with phenotypic changes between WT and ER α ^{-/-} CD11c⁺ DCs as shown by the analysis of CD11b and MHC class II (MHCII) expression (Fig. 1A and C). Whereas WT CD11c⁺ DCs were mainly composed of CD11b^{int} MHC II^{int/high} cells, CD11c⁺ cells from ER α ^{-/-} BM cultures were enriched in cells expressing higher levels of CD11b and low to intermediate levels of MHC II molecules (MHC II^{low/int}). In order to control the implication of estrogens present in standard culture medium, the pure ER antagonist, ICI_{182,780} (2 x 10⁻⁸ M) was added to the cultures at day 0, 3 and 6 (Fig. 1A and B). As expected, blocking the endogenous stimulation of ER reduced the development of DCs from WT BM cells (Fig. 1A and B). Furthermore, DCs generated under such conditions

exhibited a CD11b/MHCII phenotype indistinguishable from ER α -deficient CD11c⁺ cells (Fig. 1A). In agreement with previous works (12), similar results were obtained when DC were generated in steroid hormone-deficient medium (Fig. 1 C and D). Addition of E2 (10 nM) in cultures of WT but not ER α ^{-/-} BM cells effectively restored the capacity of the bone-marrow progenitors to generate normal numbers of DCs with the expected phenotype (Fig. 1 C and D).

Although our data demonstrate the obligatory role of ER α in promoting DC development, it has been previously suggested that ER β could also be implicated DC differentiation from BM precursors (12). To address this point, BMDCs were generated from ER β ^{-/-} or ER β ^{+/+} progenitors in steroid free medium supplemented or not with E2 (10 nM). Absence of E2 led to an impaired development of CD11c⁺ DCs in both ER β ^{+/+} and ER β ^{-/-} BM cell cultures that exhibited a CD11b/MHCII phenotype similar to ER α ^{-/-} DCs (Fig. 2 A). Addition of E2 to the steroid-free cultures allowed ER β ^{-/-} BM progenitors to differentiate into DCs as efficiently as ER β ^{+/+} or ER α ^{+/+} control cells (Fig. 2A and B). Again, E2 supplementation of ER α ^{-/-} BM cultures could not restore normal numbers of CD11c⁺ DCs in agreement with data in Fig. 1. Similar results were obtained when BMDCs were generated in conventional culture medium (CM) containing regular FCS and thereby E2 (Fig. 2C). Altogether, these data demonstrate that estrogens are required to support efficient DC development from BM precursors *in vitro* through ER α , but not ER β .

Deficiency of ER α ^{-/-} BM cells to develop into DCs is a cell-autonomous feature.

As ER α -signaling has been shown to regulate cytokine production in myeloid cells *in vitro* (18, 19), it was important to distinguish if the impaired DC development was caused by a cell-intrinsic defect of ER α -signaling within the DC lineage or by an indirect effect due to autocrine or paracrine factors which could regulate DC development. We examined the

generation of CD11c⁺ DCs from either ER α ^{+/+} or ER α ^{-/-} Ly5.2 BM cells when co-cultured with equal numbers of Ly5.1 WT BM progenitors in CM supplemented or not with an excess of E2 (Fig. 3). As shown in Fig. 3A, the proportion of CD11c⁺ Gr-1⁻ DCs expressing the CD45.2 allotypic marker was reduced by more than 2-fold when ER α ^{-/-} CD45.2 BM cells were cultured in competition with WT CD45.1 cells. This difference was even exacerbated in E2-supplemented medium, indicating that high dose of E2 further promoted DC development in WT but not in ER α ^{-/-} BM progenitors (Fig. 3B and E). In addition, analysis of the CD11b/MHC II expression profile of ER α ^{-/-} DCs (CD45.2) generated in the presence of WT CD45.1 progenitors (Fig. 3C) exhibited a similar phenotype as ER α ^{-/-} DCs generated alone (see Fig. 1). To better define the DC subsets generated under these various conditions we also assessed the relative expression of CD11b and Ly6C among CD11c⁺ cells. Indeed, E2 has been shown to preferentially promote the differentiation of CD11c⁺ CD11b^{int} lacking Ly6C expression, whereas the proportion of CD11b^{hi} Ly-6C⁺ cells among CD11c⁺ cells was increased in the absence of E2 (20). We could identify CD11b^{hi} Ly-6C⁺ and CD11b^{int} Ly6C⁻ subsets in both WT and ER α ^{-/-} DC cultures (Fig. 3D and E). The frequency of CD11b^{hi} Ly-6C⁺ cells was increased in DCs developing from ER α ^{-/-} progenitors. Similar results were obtained when DC were generated from WT BM in the absence of E2 (not shown). Ly6C⁻ CD11c⁺ cells expressing an intermediate and homogenous level of CD11b (CD11b^{int} Ly6C⁻) were the most frequent subset in the progeny of ER α ^{+/+} BM cells. By determining the number of DCs generated in each combinations, we observed that the absolute number of CD11b^{int} Ly-6C⁺ among CD45.1/CD45.2 was neither affected by the presence of a functional ER α gene in BM precursors nor by providing excess E2 during DC differentiation (Fig. 3E). By contrast, the generation of CD11b^{int} Ly-6C⁻ DCs, which represented the majority of CD11c⁺ cells from WT BM cultures was strongly dependent upon ER α -signaling. Indeed, when co-cultured with ER α ^{-/-} cells, ER α ^{+/+} (CD45.1⁺) cells represented 75% to 87% of total CD11b^{int}

Ly-6C⁻ CD11c⁺ in the absence or presence of exogenously added E2, respectively. Thus, the generation of CD11b^{int} Ly-6C⁻ DCs from ER α ^{-/-} BM precursors could not be rescued by WT hematopoietic progenitors. Reciprocally, the development of WT CD45.1 DCs was not affected by the presence of ER α -deficient BM cells. Altogether, these results further underscore a requirement for ER α activation in DC development and provide evidence for a cell-autonomous function for ER α -signaling in DC generation.

ER α -deficient DC show phenotypic and functional abnormalities.

DC development is decreased in absence of ER α -signaling but not abolished. The DC that develop in these conditions are enriched in cells expressing high levels of CD11b, Ly-6C and lower levels of MHC class II, that may represent immature myeloid DCs. ER α ^{-/-} DCs were enriched in cells expressing low to undetectable levels of MHC class II molecules and displaying high CD11b staining (see Fig. 1 to 3, and Fig. 4A). Although expression of costimulatory molecules was similar between the majority of untreated immature ER α ^{-/-} and control DCs, the frequency of cells expressing high levels of MHC class II and CD86 was higher in WT DCs than in ER α ^{-/-} DCs (Fig. 4A). DCs were stimulated through TLR4 (LPS) or TLR9 (CpG-DNA) for 24 h, followed by flow cytometric assessment of surface expression of MHC class II, CD86 and CD40 costimulatory molecules. As expected, a strong up-regulation of MHC class II and co-stimulatory molecules CD86 or CD40 was observed in WT DCs after stimulation with LPS or CpG-DNA. By contrast, despite an increased expression of CD40 molecules to levels similar to WT DCs, about 30% to 50% of ER α ^{-/-} DCs failed to upregulate MHC class II or CD86 molecules upon stimulation through TLR4 or TLR9 (Fig. 4A). Thus, as for MHC class II molecules, upregulation of CD86 was significantly impaired in some ER α ^{-/-} DCs in response to LPS or CpG. By contrast, no major defect in CD40 expression was observed after LPS- or CpG-induced maturation of ER α ^{-/-} DCs.

We also measured IL-6 and IL-12 production by DCs stimulated with poly I:C (TLR3), LPS, CpG-DNA in the presence or absence of IFN- γ . As shown in Fig. 4B, ER $\alpha^{-/-}$ DCs stimulated with LPS or poly I:C secreted more IL-6 and TNF- α (not shown) than WT DCs, whereas cytokine production in response to CpG was slightly reduced in ER $\alpha^{-/-}$ DCs (Fig. 4B). Likewise, in the presence of IFN- γ , IL-12p70 secretion was again superior in ER $\alpha^{-/-}$ DC cultures stimulated with LPS or polyI:C as compared to WT DCs. Thus, despite some defects in their maturation processes ER $\alpha^{-/-}$ DCs exhibited an enhanced capacity to produce various pro-inflammatory cytokines in response to microbial components that trigger DCs through TLR4 or TLR3. This observation was confirmed by single cell analysis of IL-6 and TNF- α production by intracellular staining after LPS stimulation. DCs producing either IL-6, TNF- α or both, were more frequent in CD11c $^{+}$ ER $\alpha^{-/-}$ DCs stimulated by LPS (Fig. 4C). This enhanced LPS-responsiveness of ER $\alpha^{-/-}$ DCs was correlated with an increased frequency of cells expressing high levels of TLR4 and CD11b molecules (Fig. 4E).

T cell stimulatory capacity of ER $\alpha^{-/-}$ DCs is impaired.

Because the principal function of DC is to activate T lymphocytes, we next evaluated the ability of ER $\alpha^{-/-}$ DCs to prime OVA-specific naive CD4 $^{+}$ T cells from DO11.10 Tg mice. For this purpose the ER α -mutation was back-crossed to B10.D2 mice to generate ER $\alpha^{-/-}$ mice of the H-2 d haplotype. The defect in BMDC development was identical between B10.D2 and C57BL/6 ER α -deficient mice (data not shown). DCs were generated from B10D2 ER $\alpha^{-/-}$ or ER $\alpha^{+/+}$ mice and CD11c $^{+}$ purified DCs were then used to stimulate transgenic DO11.10 CD4 $^{+}$ T cells which express a TCR specific for the I-A d /OVA₃₂₃₋₃₃₉ peptide complex. The proliferative capacity of DO11.10 CD4 $^{+}$ T cells was significantly impaired when ER $\alpha^{-/-}$ DCs were used as APCs in response to both OVA₃₂₃₋₃₃₉ peptide (Fig. 5A and B) or OVA protein (Fig. 5C and D). We next determined whether the defective capacity of ER α -deficient DCs to

prime OVA-specific CD4⁺ T cell proliferation was due to lack of E2-mediated signaling during DC development. B10.D2 DCs were generated in steroid-free medium supplemented or not with various doses of E2. Purified DCs were then tested for their capacity to activate DO11.10 T cells in the presence of OVA₃₂₃₋₃₃₉ peptide. As shown in Fig. 5E, WT DCs generated in the absence of E2, like ER α -deficient DCs, exhibited a reduced capacity to induce the proliferation of DO11.10 CD4⁺ T cells. This functional defect was reversed by adding exogenous E2 to WT but not to ER α ^{-/-} DCs. Indeed, when DCs were generated in the presence of doses ranging from 0.1 to 10 nM, they were able to efficiently activate naive CD4⁺ T cells (Fig. 5E and data not shown). This was confirmed by analyzing CFSE dilution in DO11.10 T cells (data not shown). E2 at 0.01 nM or below could not support efficient DCs development and DCs generated in this conditions had a phenotype similar to ER α ^{-/-} DCs (data not shown).

DCs that develop in the absence of ER-signaling have reduced cytokine response to CD40 triggering.

Because DC effector functions are markedly dependent on T cell-derived signal (5, 6), we assessed the effect of CD40 ligation on the cytokine response of WT or ER α ^{-/-} DCs. We showed that CD40 expression was similar between immature WT and ER α ^{-/-} DCs and was strongly upregulated in both DC populations upon stimulation with LPS or CpG (Fig. 4). We next evaluated the capacity of DCs to respond to CD40-dependent signaling. Culturing WT DCs on a monolayer of CD40L-expressing fibroblasts, but not control cells (not shown), induced high levels of IL-6 and IL-12p40 (Fig. 6A). In contrast, cytokine production was strongly reduced in ER α ^{-/-} DCs upon CD40 triggering (Fig. 6A). Addition of an excess of E2 during DC development resulted in an enhanced production of IL-6 and IL-12p40 in WT but not in ER α ^{-/-} DCs (Fig. 6 B). Similar results were obtained upon CD40L-stimulation in the

presence of IFN- γ (Fig. 6C). In addition to IL-6, high levels of IL-12p70 were induced in WT DCs, but not in ER $\alpha^{-/-}$ DCs. Cytokine production by DCs was blocked in the presence of anti-CD154 antibody (Fig. 6C).

We then determined the frequency of IL-12p40-producing cells by intracellular staining of DCs stimulated with CD40L-transfected fibroblasts for 18 h. Compared with baseline staining with an isotype control mAb, 10% to 25% of WT DCs could be stained for IL-12p40 (Fig. 6D and data not shown). By contrast the frequency of IL-12p40-producing cells was reduced by 3 to 10-fold in ER $\alpha^{-/-}$ DCs (Fig. 6D and data not shown). Cytokine-producing cells were contained in DCs expressing high levels of MHCII in both CD40L-stimulated WT and ER $\alpha^{-/-}$ DCs. DCs that had upregulated MHCII molecules (MHCII^{hi}) had also increased their expression of CD40 when compare to MHCII^{low} DCs. Notably, no difference were seen between WT and ER $\alpha^{-/-}$ DCs, which indicates that defective CD40L-mediated activation of ER $\alpha^{-/-}$ DCs can not be explained by a reduced expression of CD40 molecules.

Finally, we evaluated whether the functional differences we observed in ER $\alpha^{-/-}$ DCs were also found in DCs generated from WT progenitors in the absence of estrogens. Purified WT DCs generated in steroid-free medium supplemented or not with E2 were activated for 24 h with LPS (Fig. 7A and B) or CD40L-transfected cells (Fig. 7 C and D) in the absence (Fig. 7, A and C) or presence of E2 (Fig. 7, B and D). As shown in Fig. 7A, IL-6 synthesis was strongly enhanced in LPS-stimulated ER $\alpha^{-/-}$ DCs but also in WT DCs generated in the absence of E2 (E2-deprived DCs) as compared to WT DCs generated in E2-supplemented medium. When stimulated through CD40, again ER $\alpha^{-/-}$ DCs and E2-deprived WT DCs had an identical phenotype and produced significantly less IL-6 as compared to E2-supplemented WT DCs (Fig. 7C). Similar cytokine profiles were observed when DC stimulations were performed in E2-supplemented medium (Fig. 7, B and D). Thus, the presence of the hormone

at the time of TLR- or CD40-mediated stimulation had little if any effect on cytokine production by DCs. These results are consistent with an E2 action, through ER α , on precursor cells during DC development rather than on already differentiated cells.

Discussion

In the present study, we confirm that estrogens are critical to support normal DC development from BM precursors (12), and unambiguously establish that ER α , but not ER β , is required to mediate this effect. Indeed, DCs generated from WT precursors grown in steroid-free conditions were indistinguishable from DCs derived from ER α -deficient precursors, irrespective of the presence or absence of E2. ER α -deficient DCs showed an impaired capacity to upregulate MHC class II and CD86 molecules upon TLR stimulation and to activate naive CD4⁺ T cells. Failure of ER α ^{-/-} DCs to efficiently prime CD4⁺ T cells was associated with a reduced ability to produce pro-inflammatory cytokines in response to CD40L. Thus, E2-dependent activation of ER α , but not ER β , regulates critical steps involved in DC development *in vitro*.

It has been previously shown by Kovats and co-workers that estrogens were required to promote DC differentiation from bone-marrow progenitors, but the respective roles of ER α and ER β in this effect remained unresolved (12). Noteworthy, Kovats's group previously used a first generation model of ER α -targeting mice, consisting in the insertion of a Neo-cassette into exon 1 hereafter called ER α -Neo^{-/-} (21). Although the development of DCs from ER α -Neo^{-/-} mice was impaired, addition of excess of E2 restored near normal numbers of CD11c⁺ CD11b^{int} cells in the cultures, suggesting a possible compensatory role of ER β (12). The explanation of this apparent discrepancy resides most likely in the recently characterized phenotypic difference between these two mutant strains. Although the expression of the full length 66 kD isoform of ER α (p66) is abolished in ER α -Neo^{-/-} mice, two others splice variants lacking the AF-1 transactivator domain have been identified (p55, p46) which still possess a residual estrogen-dependent transcriptional activity (22-24). By contrast, in the mouse model of complete inactivation of ER α used in the present study (13, 22) E2, even in high amounts, failed to promote DC differentiation from BM progenitors, demonstrating that

ER β -signaling could not compensate for the lack of ER α . Thus, our results show for the first time that ER α is the main receptor implicated in the E2-dependent differentiation of BM progenitors into DCs *in vitro*. These data also suggest that the AF-1 transactivator domain of ER α might be dispensable for the E2-mediated effect on DC development as it was previously shown for some vascular effects of E2 (22).

Myeloid progenitors (MP) can be distinguished into several subsets according to CD34 and CD16/32 expression, among them a CD34⁺ CD16/32⁺ common precursor for both macrophages and tissue resident DCs (MDP) has been recently identified, based on the expression of CX3CR1 (25). We showed that inhibition of ER α activation in WT BM cells during DC differentiation led to a phenotype similar to that of ER α ^{-/-} cells excluding specific myeloid precursor deficiency due to lack of estrogen-signaling in ER α ^{-/-} mice *in vivo*. Moreover, it was previously shown that E2 had maximal effect at the beginning of the culture, consistent with E2 action on precursor cells (12). In addition, impaired DC development persisted when ER α -deficient progenitors were co-cultured with WT cells, indicating a cell-intrinsic requirement for ER α activation. Likewise, the generation of WT DC was not affected by the presence of ER α -deficient progenitors. Thus, default DC development from ER α ^{-/-} progenitors was intrinsic to the cells and not due to autocrine or paracrine effects of cytokines present in the microenvironments. Activated ligand-bound ER classically leads to genomic effects. Transcriptional responses to estrogens were initially recognized to depend on specific interaction of activated ER with ERE sequences in the promoter of target genes, but interaction of ER with other transcription factor complexes, like AP-1 (26) or Sp-1 (27), are common modulating mechanisms of their transcriptional activity. Although the transcription factor families AP-1 and Sp-1 are ubiquitously expressed, they are known to regulate several myeloid-specific gene expression (28, 29). Our current hypothesis is that E2-dependent activation of ER α might regulate the activation state or expression level of transcription

factors implicated in DC lineage commitment at early stages during differentiation of BM precursors (30). Interestingly, it has been recently shown that E2 acts directly on highly purified myeloid progenitors, including MDPs (25), to regulate GM-CSF-induced DC differentiation (31).

We confirmed that the development of the principal DC subtype CD11b^{int} MHC^{int} Ly6C^{neg} was primarily impaired in the absence of ER α -signaling, whereas the development of CD11c⁺ cells expressing high levels of CD11b and Ly6C and low levels of MHCII was spared. This estrogen insensitive subset might correspond to a monocyte/macrophage-like population usually present at low frequency in WT BMDC cultures (15, 32). Indeed, we observed an 2 to 3-fold increase in macrophage-like cells in ER α ^{-/-} DC cultures by cytological staining (data not shown). This observation correlated with an increased frequency of cells expressing high levels of TLR4-MD2 active complexes and CD11b in ER α ^{-/-} DCs or in estrogen-deprived WT BMDC, in agreement with previous work (20). This could explain the higher propensity of ER α ^{-/-} DCs to produce cytokines upon LPS stimulation as both TLR4 and the β 2 integrin CD11b have been shown to act in concert to positively regulate MyD88-dependent LPS signaling in macrophages (33, 34). The commitment of myeloid progenitors to DCs vs. macrophages could be therefore differentially regulated by E2-signaling under GM-CSF-induced differentiation. It has been proposed that high PU.1 activity could favor DC at the expenses of macrophage fate through the negative regulation of the macrophage specific transcription factor Maf-B (35). ER α -signaling during DC development could therefore regulate the balance between PU.1, Maf-B or others transcription factors (30), thereby modulating DC differentiation.

The capacity of DCs to respond to T-cell dependent signals is critical to initiate adaptive immune responses and drive Ag-specific CD4⁺ T cell activation and differentiation through CD40-dependent production of polarizing cytokines such as IL-12, IL-23 and IL-6

(36). Our data clearly showed that DCs generated in the absence of E2 or ER α -signaling exhibited an impaired capacity to activate naive CD4⁺ T cells as compared to DCs generated in the presence of E2. The low level of MHC II and CD86 costimulatory molecule expressed by the main CD11b^{high} DC subsets from ER α ^{-/-} or E2-deprived WT cultures can partly account for their inability to prime CD4⁺ T-cell proliferation. Additionally, we found that E2-dependent ER α activation during *in vitro* DC differentiation enhances CD40-dependent production of IL-12 and IL-6, two important polarizing cytokines that drive expansion of naive CD4⁺ T cells to the Th1 or Th17 pathway, respectively (36). By contrast, E2 treatment on already differentiated DCs during stimulation with TLR- or CD40- ligands did not significantly modify cytokine secretion profiles. Thus, despite numerous studies showing that estrogens could inhibit NF- κ B and suppress pro-inflammatory cytokine expression in myeloid cells *in vitro* (18, 19, 37), we were unable to document any significant inhibitory effect of E2 on either TLR- or CD40- dependent cytokine production by DCs. Thus differential cytokine production between DCs that developed in the absence or presence of E2-signaling is imprinted during GM-CSF-induced differentiation and therefore reflects an E2 effect on precursors or developing DCs rather than on already differentiated cells.

Generation of conventional GM-CSF-induced BMDC is usually performed in culture medium exhibiting an estrogenic activity (estrogens present in standard FCS but also the pH indicator phenol red). Interestingly, addition of wide dose range of E2 from 0.1-10 nM in steroid free medium could restore DC development and in conventional medium could further increase CD40-dependent cytokine production. Concentrations of E2 between 0.1-1 nM correspond to physiological levels of E2 found in adult female mice, during diestrus (20-35 pg/ml) and estrus (100-200 pg/ml), respectively (38), suggesting that low levels of E2 could potentially modulate immune responses *in vivo*. Indeed, we have shown that administration of E2 in castrated C57BL/6 (B6) mice resulted in a marked upregulation of antigen-specific CD4

T cell responses and in the selective development of IFN- γ -producing cells, through ER α -signaling in hematopoietic cells (39). Interestingly, E2 has been also shown to selectively enhance IFN- γ -production by NKT cells *in vivo* (40). Furthermore, E2 treatment was also shown to enhance the susceptibility to experimental autoimmune myasthenia gravis (EAMG) a Th1-dependent B cell-mediated autoimmune disease (41). Whether this effect of E2 *in vivo* is due to a direct modulation of DC development and/or function remains however to be investigated. Understanding further the impact of ER-signaling on DC biology may therefore provide new insights into the mechanisms by which sex-linked factors affect immunity and susceptibility to autoimmune diseases in women.

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Figure legends

Figure 1. ER α activation is critical for BMDC generation. ER $\alpha^{-/-}$ or ER $\alpha^{+/+}$ BM cells were grown for 9 days with GM-CSF in conventional medium (CM) with (A, B) \pm 20 nM ICI_{182,780} or in steroid-free medium (SFM) supplemented or not with 10 nM E2 (C, D). The percentages of CD11c⁺Gr-1⁻ and CD11b^{int}MHCII⁺ cells of gated CD11c⁺ cells were determined by flow cytometry and are indicated. Absolute number of DCs generated in CM (B) or in SFM (D) are reported as the mean and the SEM of five to seven independent experiments. Differences between variables were evaluated by the Mann-Whitney *U* test (**, *p* < 0.01; ns, not significant).

Figure 2. ER β activation is dispensable for BMDC differentiation. (A) ER $\beta^{-/-}$ or ER $\beta^{+/+}$ BM cells were grown for 9 days with GM-CSF in SFM \pm 10 nM E2. The percentages of CD11c⁺Gr-1⁻ and CD11b^{int}MHCII⁺ cells of CD11c⁺ gated cells were determined by flow cytometry and are indicated. Absolute numbers of DCs generated from ER $\alpha^{-/-}$ or ER $\beta^{-/-}$ BM cells in SFM (B) or in CM (C) supplemented with 10 nM E2 are expressed as the mean and the SEM of triplicate or quadruplicate cultures. Data are representative of three experiments.

Figure 3. Intrinsic expression of ER α is required to promote BMDC development. (A, B) CD45.2 BM cells from ER $\alpha^{-/-}$ or ER $\alpha^{+/+}$ mice (3 mice per group) were cultured in competition with CD45.1 BM cells at 1:1 ratio in CM alone or supplemented with 10 nM E2. (A) Percentages of CD45.1 positive and negative (CD45.2⁺, not shown) cells gated on CD11c⁺Gr-1⁻ DCs are indicated. (B) Ratio between the frequency of CD45.1 and CD45.2 DCs (defined above as CD45.1⁻ DCs) in each combinations are indicated. Data are expressed

as mean \pm SEM of three mice per group. Data were analyzed for statistical significance with a two-tailed Student's *t* test (**, $p < 0.01$). (C and D) The expression of phenotypic (CD11c, Gr-1, CD11b, MHCII, Ly6C) and allotypic CD45.1 markers was analyzed in competition experiment performed as in panel A. The percentages of CD11b^{int}MHCII⁺ (C) and CD11b⁺Ly6C⁻ or Ly6C⁺ DCs (D) among WT CD45.1 and ER α ^{-/-} CD45.2 DCs are shown. (E) The absolute numbers of CD11b⁺Ly6C⁻ and CD11b⁺Ly6C⁺ DCs generated from WT CD45.1 BM cells cultured in competition with ER α ^{-/-} or control ER α ^{+/+} CD45.2 BM cells are indicated. Cultures were performed in CM alone or supplemented with an excess of E2 as indicated. Numbers in parenthesis indicate the ratio between CD45.2 and CD45.1 DCs. Data are representative of three independent experiments.

Figure 4. ER α ^{-/-} BMDCs are phenotypically and functionally distinct from WT BMDCs.

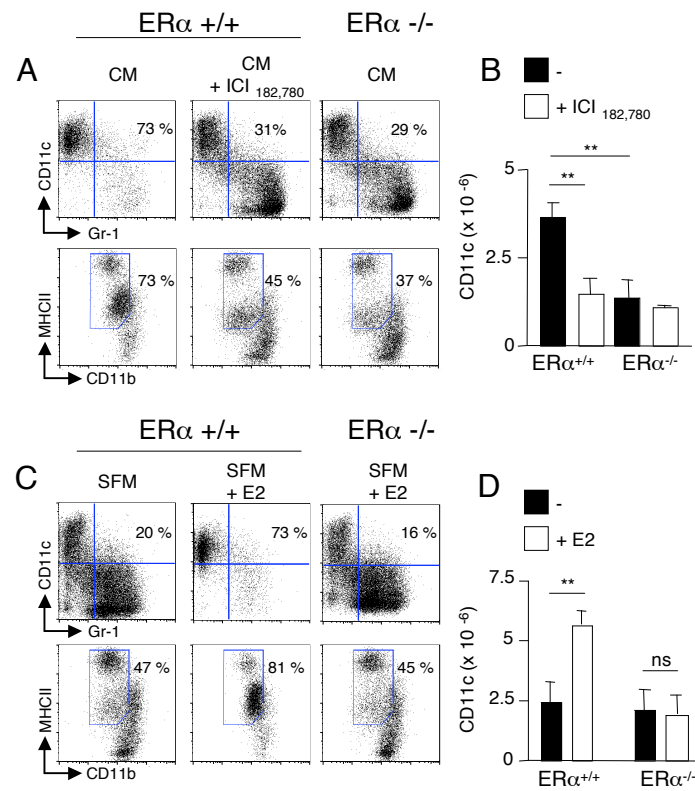
ER α ^{+/+} or ER α ^{-/-} BMDCs were generated in CM as in Fig.1 and were purified by CD11c positive selection. (A) DCs were left untreated (filled histogram) or stimulated for 18 h with TLR ligands as indicated (open histogram). Expression of MHC-II, CD86 and CD40 were analyzed by flow cytometry. (B and C) ER α ^{+/+} or ER α ^{-/-} DCs were stimulated with Poly(I:C), LPS or CpG DNA in absence (B) or in presence of IFN- γ . IL-6 and IL-12p70 were measured by ELISA in 24 h (B) or 48 h (C) culture supernatants, respectively. Results are expressed as the mean and the SEM of three mice per group. Data were analyzed for statistical significance with a two tailed Student's *t* test (*, $p < 0.05$; **, $p < 0.01$). (D) DCs were stimulated with LPS (2 μ g/ml) for 18 h and were assessed for IL-6 and TNF- α production by intracellular staining. Dot plots show IL-6 vs. TNF- α staining of gated CD11c⁺ cells. (E) Dot plots show CD11b vs. TLR-4 expression of resting CD11c⁺ ER α ^{+/+} or ER α ^{-/-} DCs. Data are representative of three to four independent experiments.

Figure 5. ER α -activation during DC development enhances the CD4⁺ T cell-priming capacity of DCs. Purified CD11c⁺ ER α ^{+/+} or ER α ^{-/-} BMDCs generated in CM as in Fig. 1 were loaded with OVA₃₂₃₋₃₃₉ peptide (A, B) or with OVA protein (C, D) to stimulate naive transgenic DO11.10 CD4⁺ T cells. Histograms show CFSE intensity of KJ1.26⁺ CD4⁺ T cells after 72 h stimulation (A,C). Results are expressed as percentage of dividing cells among KJ1.26⁺ CD4⁺ T cells (B, D) and are representative of three independent experiments. (E) BMDC cultures were conducted in SFM supplemented or not with the indicated doses of E2. ER α ^{+/+} or ER α ^{-/-} CD11c⁺ DCs were purified and were loaded with 0.1 μ M OVA peptide to stimulate DO11.10 CD4⁺ T cells. CD4⁺ T cell proliferation was assessed by ³H-thymidine incorporation during the last 12 h of culture. Results are presented as the mean and the SEM of quadruplicate cultures and are representative of three to four independent experiments.

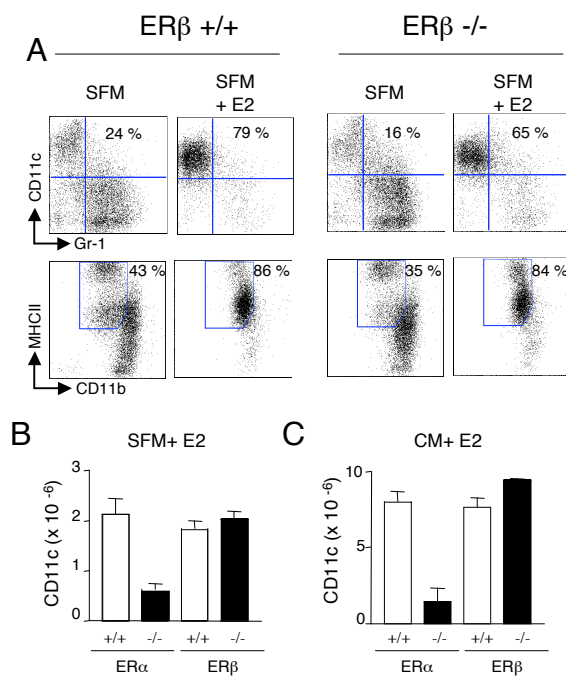
Figure 6. ER α ^{-/-} BMDCs exhibit impaired production of pro-inflammatory cytokines in response to CD40 triggering. (A) Purified CD11c⁺ BMDCs from ER α ^{+/+} or ER α ^{-/-} mice were cultured on a monolayer of CD40L-expressing fibroblasts. IL-6 and IL-12p40 were measured by ELISA in 24 h culture supernatants. Data are expressed as the mean and the SEM of four mice per group and were analyzed for statistical significance with a two tailed Student's *t* test (*, *p* < 0.05; **, *p* < 0.01). (B) E2 (10⁻⁸ M) was added or not during BMDC differentiation in CM and the production of IL-6 and IL-12p40 by CD40L-stimulated DCs was tested as above. (C) DCs (30 x 10³ DCs per well) obtained as in (B) in CM + E2 from ER α ^{+/+} or ER α ^{-/-} BM cells were cultured on a monolayer of CD40L-expressing fibroblasts with IFN- γ (5 ng/ml) to measure IL-6 and IL-12p70 production in 24 h culture supernatants. Anti-CD154 mAb was added to the cultures at the indicated doses. (D) DCs obtained as in (C) were stimulated during 18 h with CD40L-expressing cells. Cells were stained with mAb specific for CD11c, CD40 and MHC class II and fixed for intracellular analysis of IL-12p40

production by flow cytometry. Left, Dot plots show IL-12 p40 vs. MHC II of gated CD11c⁺ DC. Right, expression of CD40 on MHC II^{high} or MHC II^{low} CD11c⁺ DCs. Results are representative of two to three independent experiments.

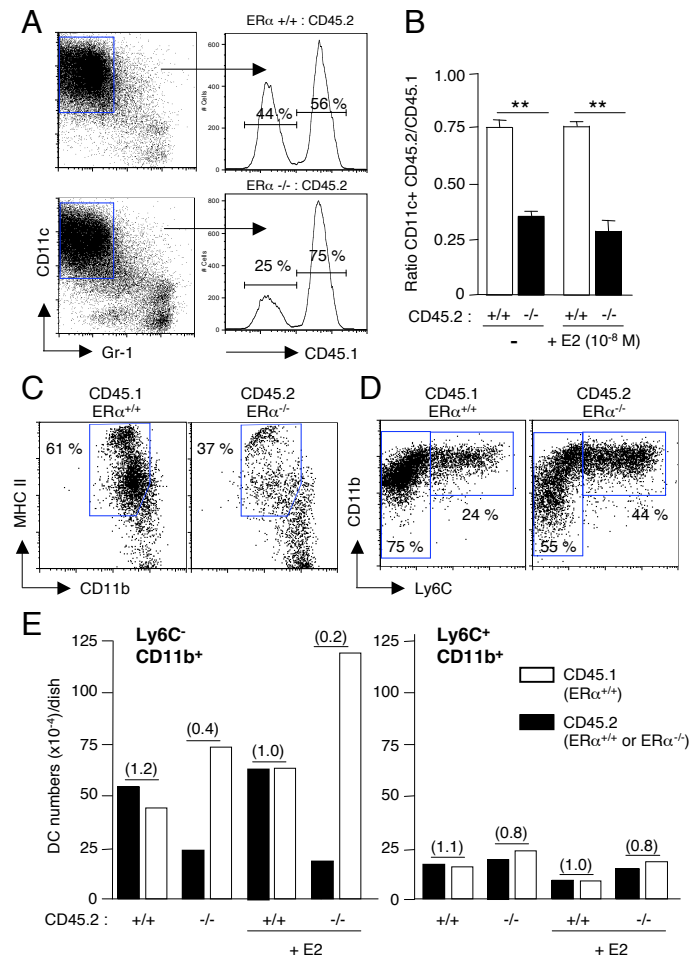
Figure 7. ER α activation during DC development controls the acquisition of specific effector functions. ER $\alpha^{+/+}$ BM cells were cultured for 9 days in SFM supplemented with 10 nM E2 (filled circle) or with vehicle (DMSO, open circle). ER $\alpha^{-/-}$ BMDCs were generated in SFM + 10 nM E2 (open triangle). Purified CD11c⁺ DCs were stimulated with LPS (A, B) or CD40L-transfected cells (C, D) in SFM (A, C) or SFM supplemented with 10 nM E2 (B, D). IL-6 production was measured in 24 h-culture supernatants by ELISA. Results are presented as the mean \pm SEM of triplicate cultures and are representative of three experiments.



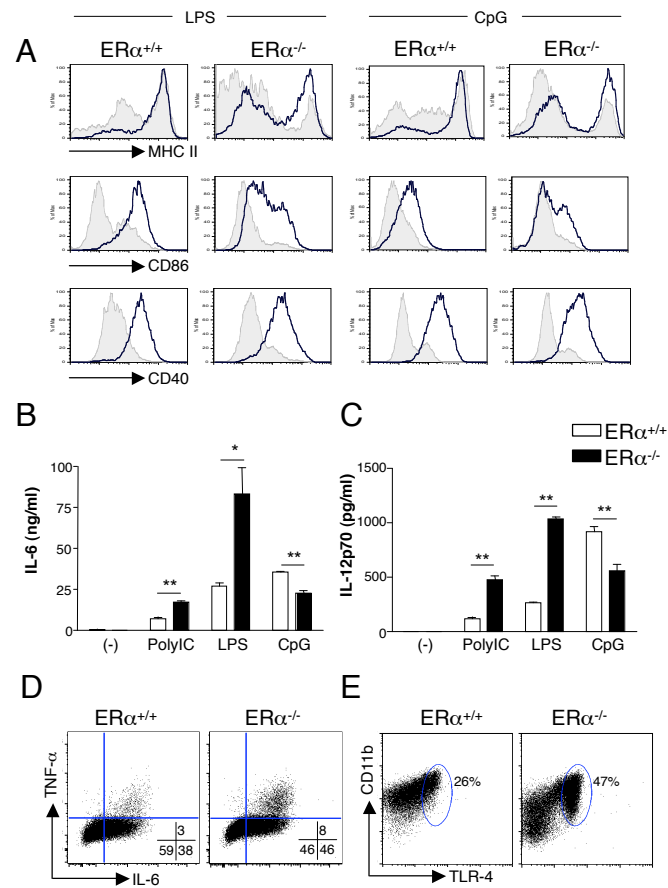
Douin-Echinard et al. Figure 1



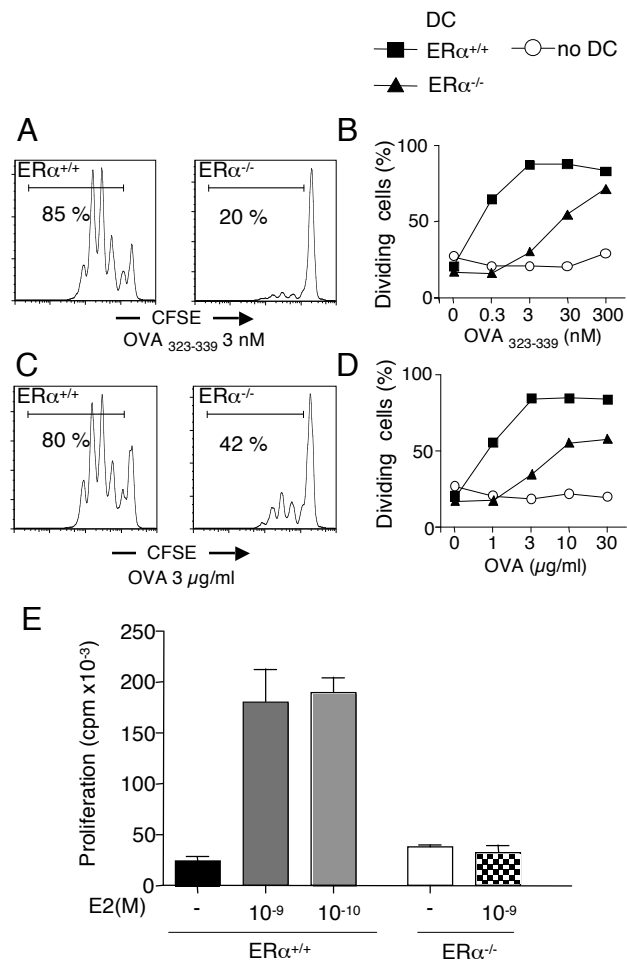
Douin-Echinard et al. Figure 2



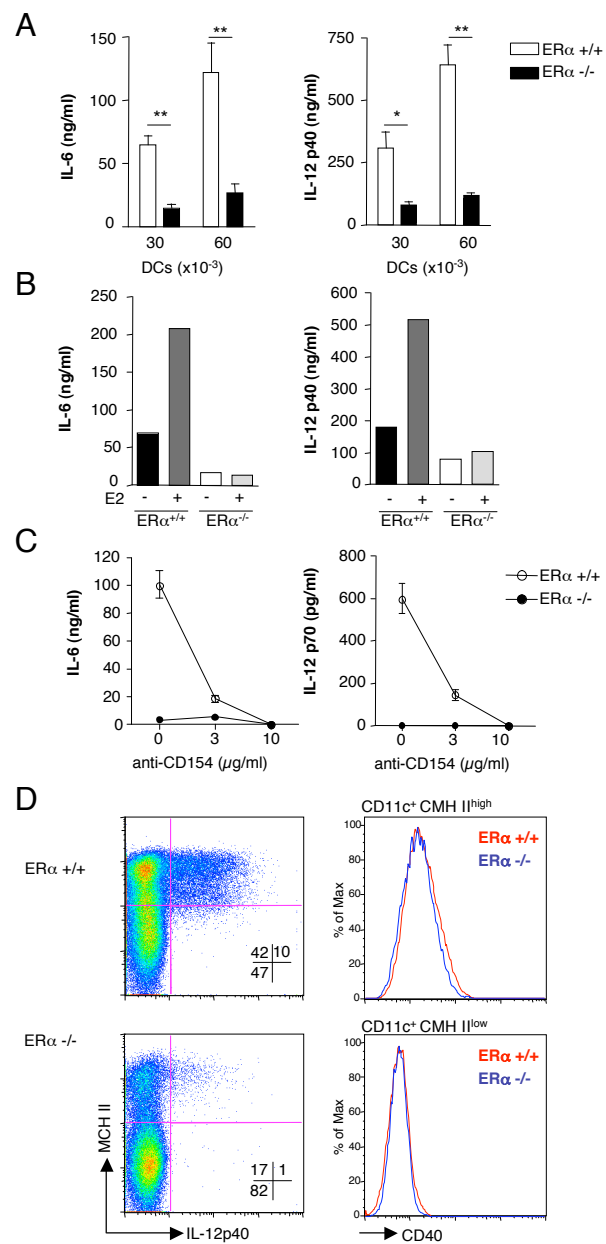
Douin-Echinard et al. Figure 3



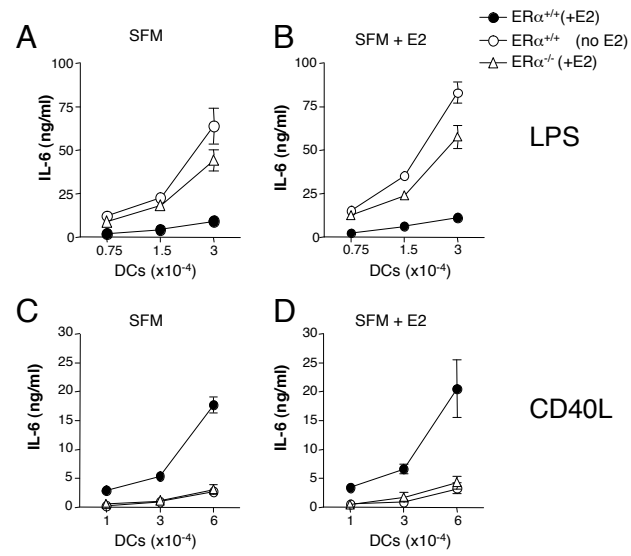
Douin-Echinard et al. Figure 4



Douin-Echinard et al. Figure 5



Douin-Echinard et al. Figure 6



Douin-Echinard et al. Figure 7